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Ribose 2′-O Methylation of the Vesicular Stomatitis Virus mRNA Cap Precedes and Facilitates Subsequent Guanine-N-7 Methylation by the Large Polymerase Protein[▽]

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During conventional mRNA cap formation, two separate methyltransferases sequentially modify the cap structure, first at the guanine-N-7 (G-N-7) position and subsequently at the ribose 2′-O position. For vesicular stomatitis virus (VSV), a prototype of the nonsegmented negative-strand RNA viruses, the two methylase activities share a binding site for the methyl donor *S*-adenosyl-L-methionine and are inhibited by individual amino acid substitutions within the C-terminal domain of the large (L) polymerase protein. This led to the suggestion that a single methylase domain functions for both 2′-O and G-N-7 methylations. Here we report a *trans*-methylation assay that recapitulates both ribose 2′-O and G-N-7 modifications by using purified recombinant L and in vitro-synthesized RNA. Using this assay, we demonstrate that VSV L typically modifies the 2′-O position of the cap prior to the G-N-7 position and that G-N-7 methylation is diminished by pre-2′-O methylation of the substrate RNA. Amino acid substitutions in the C terminus of L that prevent all cap methylation in recombinant VSV (rVSV) partially retain the ability to G-N-7 methylate a pre-2′-O-methylated RNA, therefore uncoupling the effect of substitutions in the C terminus of the L protein on the two methylations. In addition, we show that the 2′-O and G-N-7 methylase activities act specifically on RNA substrates that contain the conserved elements of a VSV mRNA start at the 5′ terminus. This study provides new mechanistic insights into the mRNA cap methylase activities of VSV L, demonstrates that 2′-O methylation precedes and facilitates subsequent G-N-7 methylation, and reveals an RNA sequence and length requirement for the two methylase activities. We propose a model of regulation of the activity of the C terminus of L protein in 2′-O and G-N-7 methylation of the cap structure.

Nonsegmented negative-strand (NNS) RNA viruses use a common strategy to express their genomes (for a review, see reference 33). Their genomic RNA is encapsidated by the viral nucleocapsid (N) protein, and it is this N-RNA complex that serves as the template for the viral RNA-dependent RNA polymerase (RdRP). The RdRP comprises a virus-encoded large (L) polymerase protein that possesses all of the enzymatic activities necessary for the synthesis, capping, and polyadenylation of the mRNA and the replication of the genome. The L protein requires an additional cofactor, a phosphoprotein (P), which is required for template recognition. Some NNS RNA viruses require additional viral proteins for authentic RNA synthesis, but those proteins do not appear to contain catalytic activities.

Vesicular stomatitis virus (VSV), the prototype of the family *Rhabdoviridae*, has long served as a model to understand RNA synthesis in the NNS RNA viruses. Purified VSV particles synthesize mRNA in vitro (3), and this can also be accomplished by using a recombinant N-RNA template purified from virus supplemented with recombinant L (rL) and rP (19, 22). During mRNA synthesis, the RdRP initiates synthesis at a 3′-proximal site to copy the viral genes in a polar and sequential manner (1, 2). In response to a specific promoter element, the RdRP initiates mRNA synthesis at a highly conserved gene start sequence, which for VSV is 3′-UUGUCNNUAG-5′ (21, 34, 35), and recognizes the cognate element in the nascent transcript to add an mRNA cap structure (23, 30, 31). Termination of mRNA synthesis is also controlled such that the VSV RdRP recognizes the sequence 3′-AUACUUUUUUU-5′ to polyadenylate the mRNA through reiterative transcription of the U tract and to terminate synthesis of the mRNA (4, 5, 14).

The mechanism by which the VSV L protein adds the mRNA cap structure is distinct from that of all other known capping reactions. Specifically, a polyribonucelotidyltransferase (PRNTase) transfers a monophosphate RNA onto a GDP acceptor through a covalent L-RNA intermediate (23). This is in contrast to other capping reactions, in which a guanylyltransferase transfers GMP derived from GTP onto a diphosphate acceptor RNA (for a review, see reference 10). It is generally thought that the capping activity of L resides within one of six conserved regions (CR) that were identified by sequence alignments (26). Amino acid substitutions in a conserved GXXT(X_{68-70})HR motif in CRV prevent mRNA cap addition, implicating CRV as the PRNTase (19). In addition to the altered mechanism of cap addition, methylation of the cap structure is also unusual in that both guanine-N-7 (G-N-7) and ribose 2′-O positions are modified via what appears to be a single methyltransferase (MTase) domain within the L protein (11, 12, 17, 20). Sequence alignments and structural predictions between known 2′-O MTases and CRVI of L identified a putative MTase domain comprising the catalytic tetrad K-D-

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K-E and a GxGxG binding site for the methyl donor *S*-adenosyl-L-methionine (SAM) (6, 9). In transcription reactions carried out using rVSV, single amino acid substitutions in this K-D-K-E motif ablate all mRNA cap methylation (11, 12, 17), whereas single amino acid substitutions within the predicted SAM binding motif either prevented all cap methylation or specifically reduced G-N-7 methylation (20). These results suggested that the two methylase activities share a single binding site for SAM and that the two reactions can proceed in an unconventional order in which 2′-O methylation occurs first. However, those experiments could not determine whether the K-D-K-E motif is specifically required for G-N-7 methylation, as the lack of cap methylation may reflect a requirement for 2′-O methylation to occur first. In contrast to that idea, experiments with Sendai virus (SeV) demonstrated that purified rL protein or a C-terminal fragment comprising CRV and CRVI or CRVI alone was capable of G-N-7 methylation of virus-specific mRNA (24). However, those experiments did not detect a ribose 2′-O methylase activity associated with the SeV L protein and therefore could not address whether the two MTase activities reside within the same region of L.

In the present study, we evaluated how the two MTase activities of the VSV L protein are regulated. We reconstituted methylation in vitro by using highly purified rL and capped RNA. This recapitulated both MTase activities independently of ongoing RNA transcription and allowed us to determine whether the methylase activities of L are coordinated. The results of this study demonstrate that the L protein functions as an efficient 2′-O MTase that facilitates a relatively inefficient G-N-7 MTase and that the K-D-K-E catalytic tetrad is essential for 2′-O methylation but also plays a role in G-N-7 methylation. We further demonstrate that the MTase activities are sequence specific and that they require a longer RNA substrate for methylation than does the PRNTase activity of L in vitro.

MATERIALS AND METHODS

Preparation of triphosphate RNA substrates. An rVSV containing a 132-nucleotide (nt) gene inserted at the *leader-N* gene junction (32) was used as the source of the 10-, 51-, and 110-nt RNAs containing the conserved elements of a VSV mRNA. The N-RNA template was purified from this virus and resuspended in 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 1 mM EDTA as described previously (18). RNA was synthesized from 10 μg of this template by using 3 μg of the cap-defective (H1227A) rL mutant as described previously (18, 19). To eliminate 5′-triphosphorylated RNA transcripts derived from the *leader* and *N* genes, we used oligonucleotides designed to anneal to nt 2 to 17 and 66 to 81 of the VSV antigenome and cleaved the resulting RNA-DNA hybrids with RNase H. Briefly, DNA primers were annealed at a 3:1 molar excess of the RNA transcripts and digested with 10 U of RNase H (Takara) for 30 min at 37°C in 60-μl reaction mixtures, followed by digestion with 1 U of RQ1 DNase (Promega) for 30 min at 37°C. A similar RNase H digestion strategy was used to generate pppRNAs of different lengths from the rVSV-132 template. Specifically, the products of in vitro synthesis from the 132-nt gene were exposed to oligonucleotides designed to anneal to positions 111 to 125, 52 to 72, or 11 to 26 of the transcript from the 132-nt gene to generate 110-, 51-, or 10-nt RNA, respectively. The 110-nt RNA was purified with an RNeasy column (Qiagen), and the 51- and 10-nt RNAs were purified by phenol-chloroform extraction. To generate the 5-nt pppRNA transcript, we used partially double-stranded synthetic DNA templates containing a T7 polymerase promoter as previously described (19, 23). A 110-nt RNA with a non-VSV mRNA start sequence (pppGGACGAAGAC) was synthesized by T7 RNA polymerase from a double-stranded DNA template. T7 transcription of this template is designed to provide a positive-sense antigenomic RNA analog of VSV containing two additional G residues at the 5′ terminus.

Preparation of capped RNA with vaccinia virus enzymes. The 5′-triphosphorylated RNA transcripts were capped in the presence of 20 μCi of [α-^{32}P]GTP (3,000 Ci/mmol; Perkin-Elmer) and 7.5 U of the vaccinia virus capping enzyme (Epicentre) in 15-μl reaction mixtures. After 2 h at 37°C, the reaction mixtures were treated with 10 U of calf intestinal alkaline phosphatase (NEB) for 1 h, followed by purification as described above. Capped RNA was quantified by measuring the incorporation of [α-^{32}P]GTP and extrapolation of molar quantities from a standard curve of moles versus counts per minute. For methylation at the G-N-7 position, 100 μM SAM was included in the capping reaction mixture. For methylation at the ribose 2′-O position or at both the G-N-7 and ribose 2′-O positions, the capped RNA or the G-N-7-methylated RNA was incubated with 100 μM SAM and 7.5 U of vaccinia virus 2′-O MTase (Epicentre).

Expression and purification of VSV polymerase. Polymerase components were expressed from recombinant baculoviruses in *Spodoptera frugiperda* 21 cells as described previously (19). The rL and rP proteins were purified by using Ni-nitrilotriacetic acid–agarose (Qiagen), followed by ion-exchange chromatography (GE Healthcare) as described previously (19).

VSV L-mediated cap methylation in *trans*. For methylation reactions, 20 fmol of capped RNA was incubated with 2 μg of rL in 15-μl reaction mixtures containing 40 mM Tris-HCl (pH 7.0), 150 mM NaCl, 0.05 mg/ml bovine serum albumin, 3% glycerol, 2 mM dithiothreitol, and 100 μM SAM for 3 h at 30°C. Reactions were stopped by the addition of 1% sodium dodecyl sulfate and 0.5 mM EDTA, the reaction products were digested with 1 μg of proteinase K for 40 min at 50°C, and the RNA was purified as described above. To analyze the cap structure, the RNA was digested with 0.3 U of nuclease P1 in 30 mM Na acetate (pH 5.3) and 0.4 mM $ZnSO_4$ at 45°C for 2 h. The products were resolved by thin-layer chromatography (TLC) on polyethyleneimine (PEI) cellulose F sheets by using 1.2 M LiCl as a solvent. Spots were visualized and quantified with a PhosphorImager (GE Healthcare). For optimization curves, morpholineethanesulfonic acid (MES)-NaOH buffers were used for pH values of 5 to 6.6, Tris-HCl for pH values of 6.8 to 8, and glycine-NaOH for pH values of 9 to 10, and the NaCl concentration, $MgCl_2$ concentration, and temperature were varied as described in Results.

RESULTS

VSV L protein possesses mRNA cap G-N-7 and ribose 2′-O MTase activities. To establish an in vitro assay for the mRNA cap MTase activities of VSV, we expressed the viral L and P proteins separately in insect cells by using baculovirus vectors and purified them by using Ni-nitrilotriacetic acid–agarose and subsequent ion-exchange chromatography (Fig. 1A). As the source of RNA, we transcribed uncapped VSV mRNA by using a cap-defective (H1227A) rL protein (19). This approach was necessary owing to low levels of transcription of RNA with the required VSV pppAACAGAUAUC sequence by T7 RNA polymerase. The low levels of transcription likely reflect the fact that the promoter for T7 RNA polymerase extends into the transcribed RNA and strongly prefers G at the +1 and +2 positions. Because cap-defective polymerases of VSV terminate transcription prematurely, we used as the template rVSV-132, which contains a short, nonessential gene inserted between the *leader* and *N* genes of VSV (32). Transcription of this template by the cap-defective L protein yields the VSV 47-nt leader RNA, the 123-nt RNA, and some N mRNA (18). To eliminate the leader and N transcripts and to precisely define the 3′ end of those transcripts from the 132-nt gene, we used oligonucleotide-directed RNase H cleavage as described in Materials and Methods. This resulted in the production of a 110-nt RNA product with an authentic VSV mRNA 5′ terminus, pppAACAGAUAUC, which was subsequently capped with the vaccinia virus capping enzyme in the presence of [α-^{32}P]GTP to generate an RNA substrate for the methylation reactions (Fig. 1B). To determine whether the VSV polymerase could methylate this RNA, we incubated 20 fmol of RNA with 2 μg of purified L, P, or L-P in the presence of 100 μM

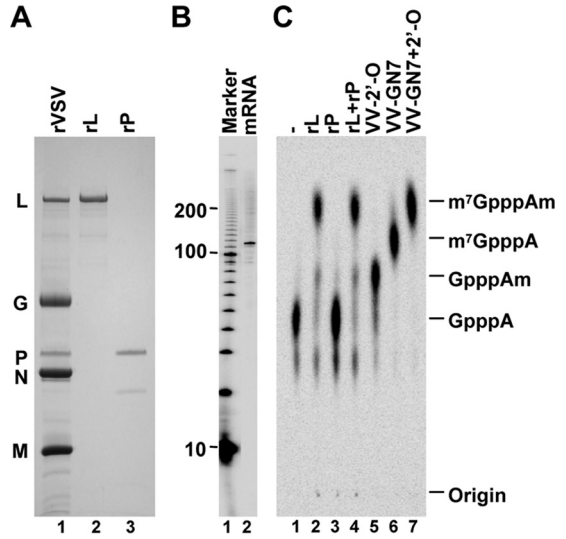

FIG. 1. Reconstitution of the mRNA cap MTase activities of the VSV L protein in vitro. (A) Baculovirus-expressed rL and rP were purified, analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and visualized following Coomassie blue staining. Lane 1, 5 μg purified VSV; lane 2, 2 μg rL; lane 3, 1 μg rP. (B) A 5′-triphosphorylated RNA containing the conserved nucleotides of a VSV mRNA start sequence was synthesized in vitro by the cap-defective (H1227A) VSV L protein from template VSV-123 containing a 123-nt gene inserted between the *leader* and *N* genes. The transcription products were purified, and the leader and N RNA transcripts were digested by oligonucleotide-directed RNase H cleavage. The transcripts of the 123 gene were similarly digested to produce a 110-nt RNA that was capped by the vaccinia virus capping enzyme in the presence of [α-^{32}P]GTP. The resulting labeled transcript was analyzed on a 6% polyacrylamide/urea gel and detected with a PhosphorImager. Lane 1 (marker [10-bp double-stranded DNA ladder from Invitrogen]) labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (NEB); lane 2, Gp*ppA-RNA. The values on the left are sizes in base pairs. (C) The Gp*ppA-RNA substrate was incubated in the presence of SAM and no enzyme (lane 1), rL (lane 2), rP (lane 3), or rL-rP (lane 4) at 30°C for 3 h. The products of the reactions were digested with nuclease P1 and then separated by TLC on PEI cellulose F sheets. Control reaction mixtures contained vaccinia virus VP39 (VV 2′-O), D1/D12 (VV G-N-7), or both, and the products were used as standards for migration of Gp*ppAm (lane 5), m^{7}Gp*ppA (lane 6), and m^{7}Gp*ppAm (lane 7), respectively. Spots were visualized with a PhosphorImager.

SAM at 30°C for 3 h. The products of this reaction were purified, digested with nuclease P1, and resolved by TLC on PEI cellulose F sheets (Fig. 1C). Nuclease P1 digestion cleaves the 3′-5′ phosphodiester bonds in single-stranded RNA but does not cleave the 5′-5′ bond of the cap structure, resulting in the release of GpppA from unmethylated RNA (Fig. 1C, lane 1). Following incubation of the RNA with purified L protein, the products of nuclease P1 digestion comigrated with a m^{7}GpppAm marker, demonstrating that L possesses both G-N-7 and 2′-O MTase activities (Fig. 1C, lane 2). The L-associated MTase activities were unaltered when L was supplied in complex with its phosphoprotein cofactor, P (Fig. 1C, lane 4). As expected, the P protein does not possess any MTase activity (Fig. 1C, lane 3). To confirm the identity of each of the products of P1 digestion, we performed methylation reactions with purified vaccinia virus 2′-O MTase VP39 and/or the G-N-7 MTase D1/D12 to yield GpppAm, m^{7}GpppA, or m^{7}GpppAm (Fig. 1C, lanes 5 to 7). These results show that the VSV L protein possesses mRNA cap 2′-O and G-N-7 MTase activities that can be studied in vitro, independently of transcription, and they further show that P protein does not affect these activities of L.

Ribose 2′-O methylation precedes and facilitates G-N-7 methylation. To determine the kinetics of the methylase activities, we monitored cap methylation over time (Fig. 2A). We found that methylation at the ribose 2′-O position precedes that at the G-N-7 position. For example, approximately 34% of the cap was methylated at the 2′-O position by 5 min, compared with only 6% at the G-N-7 position by 15 min (Fig. 2A). Methylation at the 2′-O position reached a plateau of 80% by 60 min, at which point only 30% of the cap was also methylated at the G-N-7 position (Fig. 2A). This contrasts with typical mRNA cap methylation reactions, which proceed through an obligatory order in which G-N-7 methylation occurs first. Consistent with 2′-O methylation occurring first, no monomethyl m^{7}GpppA was detected (Fig. 2A). These results suggest that ribose 2′-O methylation not only occurs with faster kinetics than G-N-7 methylation but also appears to be a prerequisite for it. To test whether the properties of the G-N-7 MTase merely reflect a requirement for a pre-2′-O-methylated RNA substrate, we used vaccinia virus VP39 to pre-2′-O methylate the RNA (Fig. 2B). Surprisingly, with pre-2′-O-methylated RNA, the kinetics of G-N-7 methylation were slower than those observed with an unmethylated RNA substrate. Specifically, after 3 h of incubation with L, only 31% of the cap was in the form m^{7}GpppAm, compared with 58% when the RNA was unmethylated (Fig. 2A and B). This demonstrates that the presence of a 2′-O methyl group on the RNA substrate does not account for the apparent preferred order of methylation and instead indicates that the act of ribose 2′-O methylation by L itself facilitates the subsequent G-N-7 methylation. We therefore tested whether this order—i.e., 2′-O prior to G-N-7—is obligatory for VSV. Using RNA that was premethylated at the G-N-7 position by vaccinia virus D1/D12, we found that the kinetics of 2′-O methylation were unaltered compared to those observed with unmethylated RNA (Fig. 2C). Taken together, these experiments demonstrate that the methylation of VSV RNA proceeds through a preferred order where modification at the ribose 2′-O methylation precedes and facilitates subsequent G-N-7 methylation.

The two MTase activities of L require similar conditions for activity. To determine the optimal conditions for each of the MTase activities, we individually altered the temperature, pH, and salt conditions of the reaction and measured the accumulation of 2′-O- and G-N-7-methylated products. Under all of the conditions tested, the 2′-O MTase was more active than the G-N-7 MTase (Fig. 3A to D); however, the 2′-O and G-N-7 MTase activities exhibited similar optima, with maximum activity at pH 7.0, 1 mM $MgCl_2$, 150 mM NaCl, and 30°C. The most noticeable difference between the activities of the two MTases occurs at alkaline pH, where G-N-7 activity appears to be more sensitive to inhibition than 2′-O activity. These data show that the 2′-O and G-N-7 MTase activities require similar conditions for optimal activity, which is consistent with the dependence of G-N-7 methylation on prior 2′-O methylation.

Sequence preference exhibited by the VSV mRNA cap MTase activities. VSV mRNAs have a 5′ sequence, pppAAC AGNNAUC, in common which acts as a *cis*-acting signal for

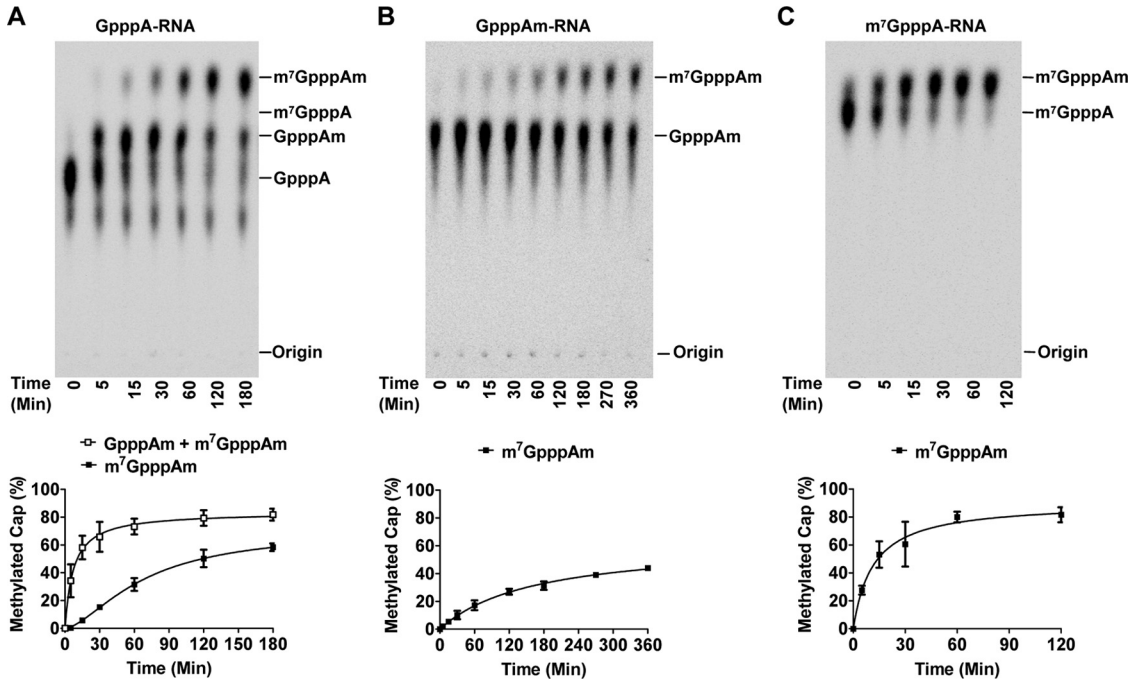

FIG. 2. Effect of the methylation status of the cap structure on the kinetics of cap methylation. (A) Twenty femtomoles of Gp*ppA-RNA was incubated with 2 μg of rL in the presence of 100 μM SAM for the indicated times, and the products were digested with nuclease P1 and then separated by TLC on PEI cellulose F as described in Materials and Methods. The Gp*ppA, Gp*ppAm, m^7Gp*ppA, and m^7Gp*ppAm spots were quantified with a PhosphorImager. The percentage of each methylated cap species was calculated as follows: (intensity of the spot corresponding to a specific methylated species of Gp*ppA/total sum of intensities of the spots corresponding to all species of Gp*ppA) × 100. The result is shown graphically as the mean of two independent experiments. (B and C) Same as panel A, except that the RNA substrate was pre-2′-O (B) or G-N-7 (C) methylated.

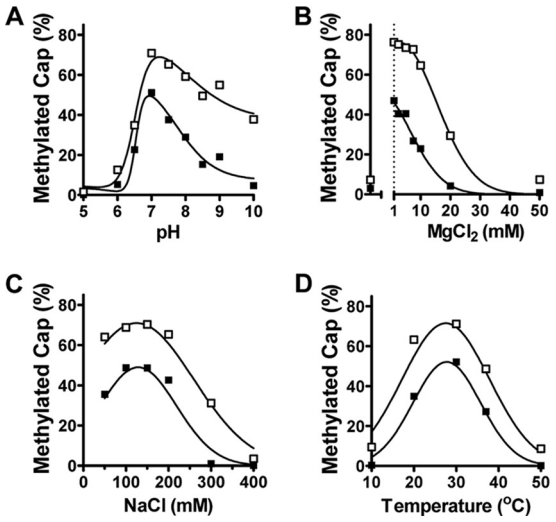

FIG. 3. Biochemical properties of the 2′-O and G-N-7 MTase activities of VSV L. Twenty femtomoles of Gp*ppA-RNA was incubated with 2 μg of rL in the presence of 100 μM SAM and varied conditions of (A) pH, (B) $MgCl_2$, (C) NaCl, and (D) temperature for 1.5 h. Each one of these parameters was varied while keeping the other three at constant optimal values. The Gp*ppA, Gp*ppAm, m^7Gp*ppA, and m^7Gp*ppAm spots were quantified with a PhosphorImager. The percentage of each methylated cap species was calculated as follows: (intensity of the spot corresponding to a specific methylated species of Gp*ppA/total sum of the intensities of the spots corresponding to all species of Gp*ppA) × 100. The percentages of 2′-O methylation (Gp*ppAm and m^7Gp*ppAm) (□) and G-N-7 methylation m^7Gp*ppAm (■) from a representative experiment are shown.

cap addition. To determine whether similar signals govern cap methylation, we tested a second 110-nt RNA with the 5′ sequence pppGGACGAAGAC (Fig. 4). We capped this RNA by using the vaccinia virus capping enzyme to yield GpppGG ACGAAGAC and performed methylation reactions. Approximately 8% of the cap structure of this mRNA was methylated, compared with 73% of the authentic VSV mRNA start (Fig. 4, lanes 2 and 4). To identify the nuclease P1 digestion products, we compared them to markers derived by using the vaccinia virus cap MTases (Fig. 4, lanes 9 to 11). These data show that the 2′-O MTase of L protein preferentially modifies authentic VSV mRNA. To determine whether the G-N-7 MTase activity also requires a specific RNA sequence, we premethylated the RNA at the ribose 2′-O position. Following 6 h of incubation, 67% of the pre-2′-O-methylated VSV mRNA was converted to the double-methylated cap, compared with only 10% of the non-VSV mRNA start RNA (Fig. 4, compare m^7GpppAm with m^7GpppGm, lanes 6 and 8). This shows that both the 2′-O and G-N-7 MTase activities of VSV L exhibit a preference for RNA that contains a VSV mRNA start sequence.

Minimal length requirement for mRNA cap methylation. To determine whether RNA shorter than 110 nt could serve as the substrate for the G-N-7 and 2′-O MTases, we synthesized transcripts that varied in length from 5 to 110 nt. For this purpose, we employed T7 RNA polymerase and synthetic DNA oligonucleotides, as previously used to generate a 5-nt substrate for the VSV mRNA capping reaction, and generated the 10-, 51-, and 110-nt RNAs by RNase H cleavage of an in

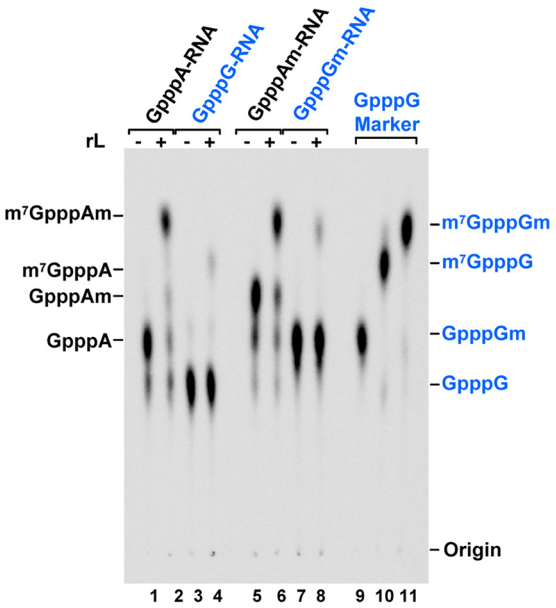

FIG. 4. The mRNA cap methylase activities require specific RNA sequence elements. Twenty femtomoles of VSV mRNA (Gp*ppA-RNA) or a T7 transcript (Gp*ppG-RNA) was incubated in methylation buffer containing 100 μM SAM and no enzyme (lanes 1, 3, 5, and 7) or 2 μg rL (lanes 2, 4, 6, and 8) at 30°C for 3 h. The RNA substrate was premethylated at the 2′-O position by using vaccinia virus VP39 (lanes 5 to 8). Standards for migration of the GpppG cap structures were generated using VV-MTases (lanes 9 to 11). The products of the reactions were digested with nuclease P1 and then separated on a TLC plate. Spots were visualized with a PhosphorImager.

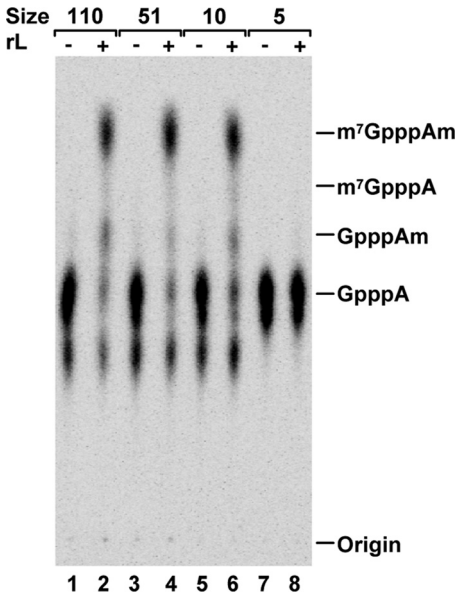

FIG. 5. Length requirement of the mRNA substrate for the MTase activity of VSV L. Twenty femtomoles of Gp*ppA-RNA consisting of 110 nt (lanes 1 and 2), 51 nt (lanes 3 and 4), 10 nt (lanes 5 and 6), or 5 nt (lanes 7 and 8) was incubated in methylation buffer containing 100 μM SAM and no enzyme (lanes 1, 3, 5, and 7) or 2 μg rL (lane 2, 4, 6, and 8) at 30°C for 3 h. The products of the reactions were digested with nuclease P1 and then separated on a TLC plate. Spots were visualized with a PhosphorImager.

vitro-transcribed VSV mRNA. Each RNA was capped by the vaccinia virus capping enzyme in the presence of [α-^{32}P]GTP and used as a substrate for methylation by VSV L. The 10-, 51-, and 110-nt RNAs served as substrates for both G-N-7 and ribose 2′-O methylations (Fig. 5, lanes 1 to 6). By contrast, the 5-nt RNA was not methylated by the VSV L protein at either the G-N-7 or the ribose 2′-O position (Fig. 5, lanes 7 and 8). Although this RNA was not methylated, we and others previously found that this RNA was efficiently capped by the VSV L protein in *trans*, and we confirmed the functionality of this specific RNA by using our *trans*-capping assay (19, 23). We conclude that the length of RNA required for methylation is larger than that required for capping by the VSV L protein in *trans*.

Single amino acid substitutions inhibit the MTase activities of L protein. CRVI of the VSV L protein contains a signature motif of 2′-O MTases that includes the catalytic tetrad K_{1651}-D_{1762}-K_{1795}-E_{1833}. We previously demonstrated that substitution of these residues abolished both 2′-O and G-N-7 methylations of mRNA synthesized by recombinant viruses (17, 20). To determine whether the inhibition of both MTase activities is a consequence of a sequential order of the two reactions, we tested the effects of substitutions at each position on methylation. We found that the substitutions K1651A, D1762A, K1795A, and E1833Q inhibited both 2′-O and G-N-7 methylations compared with wild-type L, reducing the total 2′-O methylation from 80% to less than 5% (Fig. 6B, lanes 2 to 6). In contrast, when the RNA substrate was premethylated at the 2′-O position, G-N-7 methylation was restored to between 7 and 16%, compared with 50% for wild-type L (Fig. 6B, lanes 7 to 12). These results suggest that the K-D-K-E tetrad is essential for 2′-O methylation but also plays a critical role in G-N-7 methylation which is independent of the failure of the L protein to first 2′-O methylate the RNA.

DISCUSSION

The mRNA cap methylation reactions of VSV are unusual in that the two methylase activities reside within the same protein (L) and share a single binding site for the methyl donor SAM (11, 12, 17, 20). To study the relationship between the two enzymatic activities, we established the first in vitro assay that recapitulates both 2′-O and G-N-7 methylations of the cap structure of an NNS RNA virus independently of transcription. The main conclusions of our study are that (i) 2′-O methylation precedes and facilitates G-N-7 methylation, (ii) both MTase activities are dependent upon VSV-specific sequence in the RNA substrate, and (iii) the length of the RNA substrate required for methylation is greater than that of the RNA substrate required for capping. As well as providing new mechanistic insights into the requirements for mRNA cap methylation in NNS RNA viruses, our experiments provide evidence that the same region of L protein (CRVI) is required for both ribose 2′-O and G-N-7 methylase activities. We propose a model by which the MTase activities of VSV are controlled.

Order of methylation of mRNA cap structure. The present study provides biochemical evidence that VSV L methylates its mRNA cap structure in an order that is opposite to that of cellular and most characterized viral systems. Specifically, G-N-7 methylation of VSV mRNA was exclusively detected in the

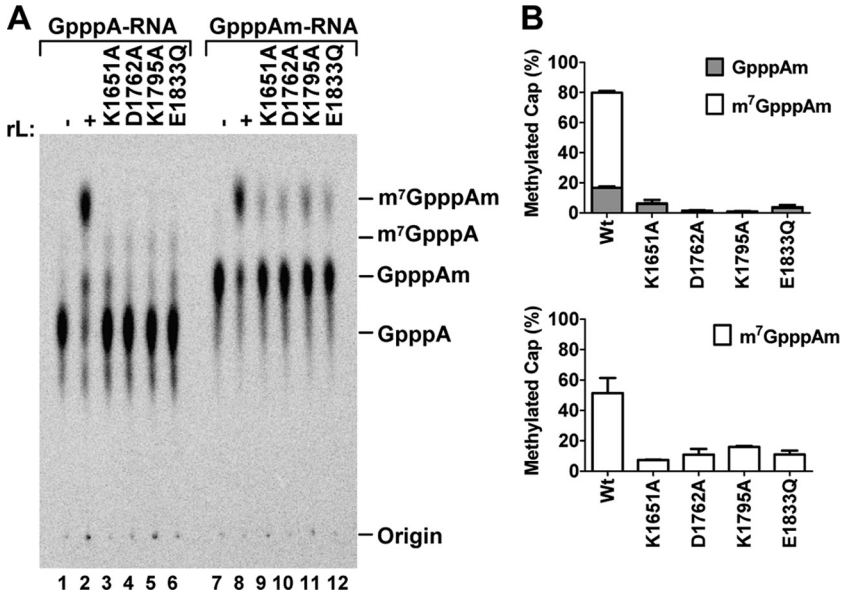

FIG. 6. Single amino acid substitutions in CRVI of L inhibit 2′-O methylation. (A) Twenty femtomoles of Gp*ppA-RNA (lanes 1 to 6) or Gp*ppAm-RNA (lanes 7 to 12) was incubated in methylation buffer containing 100 μM SAM and 2 μg of the indicated L protein at 30°C for 6 h. The products of the reactions were digested with nuclease P1 and then separated on a TLC plate. Spots were visualized with a PhosphorImager. (B) The products of methylation were quantified, and the means of three independent experiments are displayed graphically. The upper part of the panel represents the extent of methylation when the RNA substrate was unmethylated, and the lower part represents the extent of methylation following the pre-2′-O methylation of the substrate RNA. Wt, wild type.

form of a double-methylated cap and only subsequent to ribose 2′-O methylation (Fig. 2A). The precedence of 2′-O methylation over G-N-7 is not solely attributed to faster kinetics of the 2′-O MTase of L; it rather underscores a coordination of the two activities where ribose 2′-O modification precedes and subsequently facilitates G-N-7 methylation. This hypothesis is supported by the reduction of the rate of G-N-7 methylation when an exogenously pre-2′-O-methylated RNA is used as the substrate (Fig. 2B) and the lack of an effect of a pre-G-N-7-methylated substrate on the kinetics of 2′-O methylation (Fig. 2C). Since the two MTase activities appear to be carried out by CRVI of L and use the same SAM binding site (discussed below), coordination of the activities is required. This likely involves the reorientation of the relative positions of the mRNA cap and/or the SAM binding site at the end of the first methylation reaction. Since the G-N-7 position is physically upstream of the ribose 2′-O position with respect to the RNA and genetic and biochemical evidence indicates that ribose 2′-O occurs prior to G-N-7 methylation, a forward inching of the capped RNA through CRVI during ongoing RNA transcription seems unlikely to contribute to such a repositioning in an in vivo context. We speculate that a subtle intradomain change in the conformation of CRVI favoring G-N-7 methylation might be induced by the antecedent 2′-O methylation. Our observations of a decrease in the efficiency of G-N-7 methylation when an exogenously pre-2′-O-methylated substrate is used and the dramatic reduction of G-N-7 methylation by mutations in the K-D-K-E motif (discussed below) are in line with this hypothesis.

CRVI of VSV L—a dual-specificity MTase domain? Previously, we and others generated rVSV with single amino acid substitutions in CRVI of L designed to test its activity in cap methylation (11, 12, 17, 20). Substitutions in the predicted catalytic residues (K-D-K-E) ablated all mRNA cap methylation, whereas substitutions in a predicted SAM binding site (GxGxGG) either prevented all methylation or specifically inhibited G-N-7 methylation. Two conclusions were apparent from those experiments: first, that VSV mRNAs do not need to be methylated in the conventional order, and second, that CRVI appears to function as both a G-N-7 and a ribose 2′-O MTase. Here, using the *trans*-methylation assay, we found that substitutions in the K-D-K-E motif prevented both 2′-O and G-N-7 *trans* methylation. However, the substitutions retained some G-N-7 activity when the RNA was premethylated at the 2′-O position (Fig. 6A). This suggests that although the G-N-7 activity does not completely depend upon the K-D-K-E motif, those residues facilitate it. Such a role of the K-D-K-E motif in G-N-7 methylation is consistent with its conservation in members of the family *Paramyxoviridae*, such as *Newcastle disease virus*, that produce mRNA that lacks a 2′-O-methyl group (7). In members of the family *Flaviviridae*, where both G-N-7 and ribose 2′-O methylations are carried out by nonstructural protein 5 in the conventional order, the K-D-K-E motif facilitates G-N-7 MTase activity (27). We do not anticipate that the K-D-K-E motif plays a direct catalytic role in G-N-7 methylation, which typically proceeds through a chemically distinct reaction mechanism. Perhaps the motif plays a critical role in positioning the RNA substrate for the G-N-7 methylation.

Additional evidence in support of the idea that VSV L CRVI functions as a dual-specificity MTase domain is provided by the biochemical properties of the two enzymes. To a large extent, the two activities mirror one another, except at alkaline pH, where G-N-7 methylation is inhibited. We interpret this observation as reflecting that G-N-7 methylase and

2′-O methylase activities involve shared but subtly distinct features of CRVI. Another possibility is that the two MTase activities of CRVI may be differentially affected by other regions of L through allostery. We have already demonstrated that single amino acid substitutions in CRII and CRIII of L can affect the extent of cap methylation (16), although we have no evidence that these regions of L participate directly in the methylation reactions. Further experiments are required to test whether the activities of CRVI are indeed regulated through an allosteric mechanism.

Sequence-dependent mRNA cap methylation. Sequence-dependent G-N-7 methylation was previously reported for SeV mRNA (24) and followed from the demonstration that an aspect of VSV cap addition required specific sequences present within the conserved gene start element (30). The two mRNA cap methylase activities of the *Flaviviridae* have also been shown to require distinct specific *cis*-acting signals in the RNA (8). In the present study, we found that both MTase activities of VSV are also sequence specific. The extent of sequence conservation at the 5′ ends of VSV mRNAs is restricted to the first 10 nt (pppAACAGNNAUC). In *trans*-capping assays using the 5-nt substrates, substitutions at positions 1, 2, 3, and 5 were shown to diminish mRNA cap addition (23). This is in good agreement with results from detergent-activated virus transcription reactions, although position 5 substitutions were more tolerated for capping under those conditions (31). In contrast to *trans* capping, a 5-nt substrate was not sufficient for *trans* methylation, perhaps reflecting a role for conserved positions 8, 9, and 10 in mRNA cap methylation. Previously, we discriminated the requirement of the VSV gene start for initiation and cap addition (31), but we did not evaluate the role of this sequence in cap methylation. Therefore, we cannot know to what extent the signals required for each of the steps of capping overlap one another. This work, however, underscores the highly multifunctional nature of the VSV gene start site. These 10 nt contain a key *cis*-acting regulatory element for the initiation of mRNA synthesis (29, 30, 34), they contain a signal in the nascent mRNA strand that is required for mRNA cap addition (23, 30, 31), and they contain a signal in the nascent mRNA strand that is required for mRNA cap methylation.

Minimal RNA chain length for mRNA cap methylation. Earlier studies with detergent-activated VSV demonstrated that capping is intimately linked with transcription and short uncapped transcripts that correspond to the 5′ ends of mRNAs cannot be chased into capped transcripts during in vitro transcription reactions (15). This is in marked contrast to other viral and cellular systems, where the capping machinery responds to exogenously added RNA, thus facilitating analysis of the reactions (28). Studies on the polymerase of the New Jersey serotype of VSV suggested the presence of a "methylation window" in which transcripts of a certain size become accessible to the MTase, though this apparent size requirement was not mapped (13). Such a suggestion is compatible with a modular domain structure for polymerase whereby the active site for ribonucleotide polymerization present in CRIII of L is spatially separated from sites involved in the modification of the 5′ end of this transcript. Such a domain structure of polymerase would suggest that a transcript needs to have a minimal length to gain access to RNA-modifying domains within the L protein. While there is no direct evidence in support of this hypothesis, observations from in vitro transcription studies suggest that transcripts of less than 37 nt are not capped (25).

As mentioned above, a 5-nt substrate RNA is capped but not methylated by VSV L protein in *trans* (19, 23). While the different substrate length requirements for capping versus methylation are intriguing, it is not clear how such observations relate to the events that occur during cotranscriptional modification of the viral mRNA either in cells or in vitro. In contrast to the length requirement for VSV methylation, SeV L protein catalyzes G-N-7 modification of a 5-nt RNA in *trans*. Intriguingly, although CRVI of SeV L protein shows all of the hallmarks of a 2′-O MTase, 2′-O methylation was not detected (24). In addition to a difference in the substrate length, the biochemical properties of the SeV G-N-7 MTase differ from those of VSV with regard to the optimal pH and NaCl concentration. The VSV MTase activities are both optimal at physiological pH and NaCl concentrations, whereas the SeV enzyme displayed maximal G-N-7 activity at pH 6.0 and was essentially inactive at physiological NaCl concentrations (24). Given the range of conditions tested, it seems unlikely that adjusting the conditions of the reaction will result in a gain of 2′-O methylase activity for the SeV L protein. Perhaps the length of the RNA substrate is responsible for the lack of 2′-O methylation seen with the SeV L protein.

A model for mRNA methylation by CRVI of the VSV L protein. Our findings support the following model of VSV L protein-mediated cap methylation. First, in response to a specific RNA sequence, CRVI of L protein modifies the cap structure at the 2′-O position to produce GpppAmACAG-RNA. The by-product of the reaction, *S*-adenosyl-homocysteine, is presumably released prior to the binding of a second molecule of SAM and subsequent methylation of the RNA at the G-N-7 position. Our data show that the act of methylation at the 2′-O position favors the modification at the G-N-7 position of the RNA, and we interpret this finding to indicate that the RNA molecule is not released from the polymerase following the initial modification at the 2′-O position. One argument in favor of this is that the efficiency with which a pre-2′-O-methylated RNA is G-N-7 modified is reduced compared to the unmethylated RNA. We therefore suggest that CRVI exists in subtly different states. An initial probably relaxed state favors binding of unmethylated GpppRNA and the methyl donor SAM. Methylation of RNA by CRVI of L at the 2′-O position perhaps induces a conformational change that facilitates the repositioning of the RNA for subsequent G-N-7 methylation and/or favors the release of *S*-adenosyl-homocysteine and the binding of a subsequent molecule of SAM. Thus, we propose a homotropic allosteric regulation of the CRVI MTase activities. Ongoing experiments will test this model and will provide further insights into the mechanism of regulation of CRVI.

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